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Author: Snezana Agatonovic-Kustrin David W. Morton Petar Ristivojević

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Assessment of antioxidant activity in Victorian marine algal extracts using high performance thin-layer chromatography and multivariate analysis

Snezana Agatonovic-Kustrin[a], David W. Morton[b*], Petar Ristivojević[c]

[a]Faculty of Pharmacy, Universiti Teknologi MARA, Puncak Alam Campus, Bandar Puncak Alam, Selangor, 42300, Malaysia
[b]School of Pharmacy and Applied Science, La Trobe Institute for Molecular Sciences, La Trobe University, Edwards Rd, Bendigo, 3550, Australia
[c]Innovation Center of the Faculty of Chemistry Ltd, Studentski trg 12-16, 11000 Belgrade, Serbia
*Corresponding author. Tel.: +61 3 5444 7367.
E-mail address: d.morton@latrobe.edu.au (D. Morton)

Highlights

- A HPTLC method to screen marine algae for antioxidant activity has been developed
- DPPH• assay was used to establish their ability to scavenge stable free radicals
- Derivatization with $FeCl_3$ was used to establish phenolic content
- Samples were classified into 5 groups with different chemical/antioxidant profiles
- Certain brown algae show significantly higher antioxidant activities
- Phenolics, including flavonoids, are the main contributors of antioxidant activity

Abstract

The aim of this study was to develop and validate a rapid and simple high performance thin layer chromatographic (HPTLC) method to screen for antioxidant activity in algal samples. 16 algal species were collected from local Victorian beaches. Fucoxanthin, one of the most abundant marine carotenoids was quantified directly from the HPTLC plates before derivatization, while derivatization either with 2,2-diphenyl-1-picrylhydrazyl (DPPH•) or ferric chloride ($FeCl_3$) was used to analyze antioxidants in marine algae, based on their ability

to scavenge non biological stable free radical (DPPH•) or to chelate iron ions. Principal component analysis of obtained HPTLC fingerprints has classified algae species into 5 groups according to their chemical/antioxidant profiles. The investigated brown algae samples were found to be rich in non-and moderate-polar compounds and phenolic compounds with antioxidant activity. Most of the phenolic iron chelators also have shown free radical scavenging activity. Strong positive and significant correlations between total phenolic content and DPPH radical scavenging activity showed that, phenolic compounds, including flavonoids are the main contributors of antioxidant activity in these species. The results suggest that certain brown algae possess significantly higher antioxidant potential when compared to red or green algae and could be considered for future applications in medicine, dietary supplements, cosmetics or food industries. *Cystophora monilifera* extract was found to have the highest antioxidant concentration, followed by *Zonaria angustata*, *Cystophora pectinate*, *Codium fragile*, and *Cystophora pectinata*. Fucoxanthin was found mainly in the brown algae species. The proposed methods provide an edge in terms of screening for antioxidants and quantification of antioxidant constituents in complex mixtures. The current application also demonstrates flexibility and versatility of a standard HPTLC system in the drug discovery. Proposed methods could be used for the bioassay-guided isolation of unknown natural antioxidants and subsequent identification if combined with spectroscopic identification.

Keywords: Free radical scavengers, high performance thin layer chromatography, marine algae, polyphenolics, principal component analysis.

1. Introduction

Due to their long and diverse evolutionary backgrounds, marine organisms offer vast genetic diversity and present a valuable source of bioactive compounds. Marine algae, although simple chlorophyll containing organisms, have extremely diverse morphological and reproductive features and produce a range of compounds with unique physiological and biochemical properties [1]. They are also able to grow in environmental extremes, where exposure to extreme light and high oxygen concentrations, leads to the increased formation of reactive oxygen species (ROS) [2]. Despite their exposure to these harmful ROS, healthy algae lack oxidative damage in their structural components (i.e. fatty acids), indicating the presence of protective antioxidant components in their cells (vitamins, pigments, and polyphenols) [3-5] that may also offer protection to the human body against ROS.

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide are natural byproducts of normal oxygen metabolism. Under normal circumstances, cells are able to defend themselves against ROS damage using enzymatic antioxidants such as catalase, superoxide dismutase, and glutathione peroxidase [6], and non-enzymatic antioxidants, like vitamins C and E and glutathione. However, ROS levels may increase beyond concentrations that can be compensated by the production of antioxidants, resulting in oxidative damage of cellular components, leading to the cell death and tissue injury. This is associated with the onset of a variety of chronic diseases in humans, including certain cancers [7] and inflammatory diseases [6]. Hence, consumption of antioxidants might help to neutralize these excess free radicals produced in the body. Current evidence strongly supports the contribution of phenolic compounds present in the diet, in the prevention of cardiovascular diseases (CVDs) and cancers, and also suggests they play a role in the prevention of neurodegenerative diseases and diabetes mellitus [8]. While the antioxidant benefits associated with the consumption of various terrestrial plants has long been accepted, the health benefits of consuming marine algae has not been widely recognized in Western counties.

Most polyphenols isolated from marine sources are from macro- and microalgae [9]. The structures of natural polyphenols vary from simple molecules, such as phenolic acids and other simple polyphenolic compounds, to the more complex phlorotannins, which consist of polymeric structures made up of units of phloroglucinol (1,3,5-trihydroxybenzene), which are found in *Phaeophyceae* (brown algae) [10,11]. Polyphenols exhibit a wide range of biological effects due to their antioxidant properties. Many of these phenolic compounds provide a

chemical defense mechanism against predators. Moreover, the relatively high concentration of phenolic compounds in marine algae species contributes to their beneficial antioxidant properties. It is important to note that the antioxidant properties of many marine algae have been related to potential anti-aging, anti-inflammatory, anti-bacterial, anti-fungal, cytotoxic, anti-malarial, anti-proliferative, and anti-cancer effects [12,13].

Relatively little is known about the antioxidant properties of compounds derived from Victorian algae. Although there are publications on the antioxidant activity of numerous algal species commonly found in Australia, there are no reports of systematic testing for antioxidant activity. Spectrophotometric assays are commonly used for determination of total antioxidant activity and include; free radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical, nitric oxide scavenging assay, superoxide free radical scavenging assay, and hydrogen peroxide radical scavenging. However, the disadvantage of these spectrophotometric methods is that they measure the total antioxidant capacity of the whole extract and not the antioxidant activity of the individual components present in the extract [14,15]. The time consuming isolation of individual compounds can be avoided if this type of assay is combined with chromatographic separation, either high performance liquid chromatography (HPLC) or thin layer chromatography (TLC). However, the use of an on-line HPLC method has been reported not to be successful as slow reaction kinetics results in inaccurate measurements [16]. In contrast, the use TLC overcomes the disadvantages associated with the slow kinetics issue associated with the use of HPLC. When using TLC, many samples can be run simultaneously on the same plate, and therefore under the same experimental conditions, making analysis times short and reducing the cost.

TLC combined with DPPH• assay *in situ* has been previously been used for the screening of antioxidants in marine bacteria [17], plant extracts [18], wine extracts [19,20], and herbal extracts [21]. In the TLC-DPPH• assay, a developed plate is sprayed or dipped in an alcohol DPPH• free radical solution. Resulting yellow spots against a purple background indicate the presence of an active antioxidant compound [18]. Compound identification can be achieved by either fingerprinting (analyzing the thin layer chromatogram) or by subsequent analysis using instrumental techniques (i.e. mass spectrometry).

The aim of this work was to develop a simple, fast method for screening algae extracts for antioxidant activity using the TLC method, combined with post-chromatographic derivatization with either $FeCl_3$ or DPPH• free radical, in order to quantify and compare both polyphenolic content and free radical scavenging activity. Principal component analysis (PCA) was applied to extract the features from the plate image (i.e. from sample fingerprints)

and to provide full analytical information about chemical composition, similarity/dissimilarity between samples, and identified characteristic markers. PCA is a commonly used multivariate technique that is used to reduce multidimensional data set to 2D or 3D coordinates. PCA visualizes and classifies samples according to similarity, determines objects showing different properties from others (outliers), and defines important variables that can be used for data classification.

2. Material and Methods

2.1. Chemicals used

2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) free radical, iron(III) chloride (97%), fucoxanthin (98%), and gallic acid (97%), were purchased from Sigma-Aldrich (Munich, Germany). All other solvents and chemicals used were of analytical grade. Acetic acid, acetone and methanol were purchased from Merck (Darmstadt, Germany), n-hexane from BDH (Poole, England), and ethyl acetate from Sigma-Aldrich (Munich, Germany). Separations were performed on 20 x 10 cm normal phase Silica gel 60 F254 HPTLC glass plates (Merck, Darmstadt, Germany).

2.2. Sample collection and preparation

Marine algae were collected from Torquay beach, Victoria, Australia, and transported in cooled insulated containers. Within six hours of collection, algae samples were thoroughly rinsed three times with filtered seawater. Samples were then divided into 50–200 g portions, frozen and then freeze dried using a freeze dryer (Dynavac, FD12, Belmont, Australia). An ethanolic extraction was performed on 10 g of finely ground freeze dried sample using either the Soxhlet extraction method or the shake extraction method. In the Soxhlet extraction method, the sample was placed into a 22 x 80 mm cellulose extraction thimble (Whatman, Little Chalfont, UK), and refluxed in a Soxhlet apparatus for 4 hours using 100 mL of absolute ethanol. The shake extraction method, involved vigorous shaking of the sample for 15 minutes, with 100 mL of absolute ethanol in a sealed glass stoppered conical flask. The resulting solution extracts obtained with either extraction procedure were concentrated to approximately 20 mL using a rotary evaporator (Buchi Rotavapor Model R-200, Flawil,

Switzerland), transferred into a 25 mL volumetric flasks and adjusted to volume with absolute ethanol. All extracts were stored at 4 °C to minimize degradation.

Table 1

Algae samples used in this work.

Sample number	Species	Type of algae
1	*Cystophora monilifera*	brown algae
2	*Phyllospora comosa*	brown algae
3	*Cystophora pectinate*	brown algae
4	*Phacelocarpus peperocarpos*	red algae
5	*Euptilota articulata*	red algae
6	*Codium fragile*	green algae
7	*Dicaranema revolutum*	red algae
8	*Nizymenia furcata*	red algae
9	*Acrocarpia paniculata*	brown algae
10	*Perithalia caudate*	brown algae
11	*Zonaria angustata*	brown algae
12	*Cystophora pectinate*	brown algae
13	*Cystophora platylobium*	brown algae
14	*Amphibolis antartica*	sea-grass
15	*Dictyota furcellata*	brown algae
16	*Ecklonia Radiata*	brown algae

2.3. High performance Thin Layer Chromatography

HPTLC plates were pre-washed before use with a blank run of ethanol, then dried and activated, by heating in an oven at 105 °C for 15 minutes. Samples were sprayed as 8 mm wide bands using a 100 µL HPTLC syringe (Hamilton, Bonaduz, GR, Switzerland) with a semi-automatic sample applicator (Linomat 5, Camag, Muttenz, Switzerland), 8 mm from the lower edge, with 10 mm distance from each side, and a distance of 4 mm between each tracks.

2.3.1. Post-chromatographic derivatization

A freshly prepared 2% (w/v) ferric chloride solution was neutralized by adding a few drops of diluted sodium hydroxide solution until some ferric hydroxide precipitated. The solution was then filtered to remove the precipitate and the clear solution was used for derivatization [22].
A 0.4% (w/v) ethanolic DPPH• solution was used for post-chromatographic derivatization. Both solutions were stored at 2-8 °C and protected from light.

2.3.2. HPTLC plate development and chromatographic band visualization

Chromatographic plates were developed in an Automated Multiple Development Chamber (AMD 2, Camag, Muttenz, Switzerland) using *n*-hexane; ethyl acetate; acetic acid (20:10:1) as mobile phase. Images of the developed plates, were recorded using a TLC-visualizer (Camag, Muttenz, Switzerland) equipped with a 12-bit charged couple device (CCD) digital camera and winCATS software (Camag, Muttenz, Switzerland) under UV light at 366 nm and white light above and below the plate. Developed plates were photographed before and after derivatization with either $FeCl_3$ or 0.4% w/v DPPH• solution. Plates derivatized with DPPH• solution, were stored in dark for 30 min and then photographed. WinCATS image capturing parameters were fixed to ensure high quality images and reproducibility between plates. Quantitative HPTLC analysis was performed using VideoScan Digital Image Evaluation software (2003, Camag, Muttenz, Switzerland) and set to recognize fluorescent bands.

2.4. Method validation

The method was validated according to the current International Conference on Harmonization (ICH) guidelines [23]. The method was assessed based on linearity, specificity, precision, limit of detection (LOD), and limit of quantification (LOQ). The working range for determination of gallic acid, after post-chromatographic derivatization with either $FeCl_3$ or DPPH•, and fucoxanthin without post-chromatographic derivatization, was assessed by plotting chromatographic peak areas against applied amounts of standards. Linear ranges were established using the least squared method. Specificity was assessed by the ability of the optimized mobile phase to separate samples. Repeatability was assessed by

applying three repetitions of each standard at three concentrations within the calibration curve. Variance between repetitions was expressed as a relative standard deviation (%RSD).

The sensitivity of measurements of the methods used to determine total polyphenolic content, free radical scavenging activity, and fucoxanthin quantification was estimated in terms of the limit of quantitation (LOQ) and the limit of detection (LOD), the lowest concentration detected. LOQ and LOD were calculated by the use of equations LOD = 3 × *Sd*/*B* and LOQ = 10 × *Sd*/*B*, where *Sd* is the standard deviation of the peak areas of the standards (n = 3), taken as a measure of noise, and *B* is the slope of the corresponding calibration curve.

2.5. Multivariate analysis

Images of the HPTLC chromatograms were processed with the free Java based ImageJ software. (1.48c version, Wayne Rasband), an image processing program developed at the National Institute of Health, USA (http://rsb.info.nih.gov/ij). Principal component analysis was carried out by PLS ToolBox v.6.2.1, for MATLAB 7.12.0 (R2011a) [11]. Median filter (3 pixels) was used in order to remove noise. The pixel intensity which carries information about chemical composition of each algae was combined to form a data set consisting of 16 rows (number of algae samples) and 675 columns (number of pixels per sample). The data were additionally pre-processed by using mean centring, which is the preferred option when the classification of the samples is based on variables that are all measured in the same unit. PCA was carried out as an exploratory data analysis by using a singular value decomposition algorithm and a 0.95 confidence level for Q and T^2 Hotelling limits for outliers [24]. Correlation Optimized Warping (COW) was employed to correct the inter- and intra-plate peak shift due to variations in mobile phase composition, humidity, temperature, operator handling and instrumental instability [25].

3. Results and Discussion

Most phenols, when treated with neutralized ferric chloride solution, form intense red, blue, purple, or green colored complexes with the Fe^{3+} ion [26]. The appearance of one of these colors is taken as a positive test that indicates that a phenol is present in the sample. After post-chromatographic derivatization with the Fe^{3+} ion, several distinct fingerprint patterns of algae were observed according to Fig. 1. Algae samples 2, 3, 4, 6, 11, 12 and 14 were shown

to have particularly rich chemical profiles and to contain a number of antioxidant compounds. The total phenolic contents in each algal extract was determined as the sum of all color band areas and was expressed in gallic acid equivalents (GAE), by using gallic acid as a model analyte (Table 2). Statistical parameters of correlation are presented in Table 3. Good repeatability of the method was confirmed by calculating the coefficient of variation for five replicates at three different concentrations (low, medium and high concentration) of standards within the linear calibration range (Table 3). Averaged coefficients of variations were 4.2%.

Table 2

Total polyphenolic content determined using post-chromatographic derivatization with neutral ferric chloride solution, free radical scavenging activity in algal samples estimated with HPTLC-DPPH• assay and amount of fucoxanthin present.

	Polyphenolic content		Free radical scavenging activity		Fucoxanthin	
sample	Area (pixels)	GAE (µg/10 µL)	Area (pixels)	GAE (µg/10 µL)	Area (pixels)	Found (µg/10 µL)
1	112749	5.7	44120	0.6	7555	1.5
2	776309	44.1	457613	13.4	14041	2.5
3	449832	25.2	158017	4.1	69273	10.7
4	283446	15.6	125314	3.1	0	0
5	92501	4.5	13828	0.0	0	0
6	331644	18.4	38878	0.4	0	0
7	69207	3.2	64519	1.2	0	0
8	94028	4.6	620	0.0	2557	0.8
9	198463	10.7	1885	0.0	4499	1.1
10	28669	0.8	32704	0.2	1918	0.7
11	635197	35.9	328343	9.4	74591	11.6
12	189631	10.1	118071	2.8	31870	5.1
13	134035	6.9	61518	1.1	4084	1.0
14	367826	20.4	43631	0.5	0	0
15	66717	3.0	15884	0.0	4366	1.1
16	14607	0.0	7050	0.0	2522	0.8

Table 3. Accuracy and precision of the methods for the determination of gallic acid and fucoxanthin.

(n = 5).

Standard	Method derivatization	Applied/band (µg)	Found (µg)	Mean recovery (%)	RSD (%)
Gallic acid	$FeCl_3$	1.0	0.79	79.09	16.4*
		2.0	2.19	109.3	4.3
		5.0	4.95	99.07	4.1
Gallic acid	DPPH•	1.0	0.88	88.00	7.69
		3.0	3.09	102.89	4.92
		5.0	4.83	96.47	5.46
Fucoxanthin	under visible light	1.0	1.18	118.00	4.39
		3.0	2.79	92.98	4.69
		5.0	5.16	103.33	3.95

*Below limit of quantification

Table 4

Linearity, LOD, and LOQ for the determination of gallic acid after post-chromatographic derivatization either with $FeCl_3$ neutral solution or DPPH• and for the direct determination of fucoxanthin under white light.

Standard	Method	Equation of the line	R	Linear range	t_{calc}	t_{tab}	LOQ (µg)	LOD (µg)
Gallic acid	$FeCl_3$	$y = 12255x + 26397$	0.95	1.0-10.0	3.69	2.37	1.29	0.39
Gallic acid	DPPH	$y = 32284x + 26460$	0.98	0.5 - 8.0	2.96	1.77	0.4	1.8
Fucoxanthin		$y = 6701x + 2589$	0.99	0.5-8.0	0.89	1.78	0.3	1.0

x = applied amounts (µg); y = band area (pixels); R = correlation coefficient; t_{cal} = calculated t value; t_{tab} = tabular t value at a = 0.05.

Free radical scavenging activity of investigated extracts was assessed using a direct DPPH• assay. DPPH• is a stable, deep purple colored free radical that turns into pale yellow when reduced by antioxidants present in the sample. Therefore, antioxidants from the sample appear as yellow spots against a purple background on the plate (Fig. 2c).

Results of the DPPH• assay show large variation in free radical scavenging activity among the different algal species. Green algae and red algae have lower free radical scavenging activities when compared to brown algae. The total area size of the yellow bands obtained after the DPPH assay for some brown algae extracts (samples 2, 3, 11 and 12) exceeds many times the size of the yellow band area obtained with the highest activity red algae sample (sample 4).

Fig. 1

The degree of free radical scavenging activity in extracts was expressed in gallic acid equivalents (GAE) by comparing the area size and color intensity of the yellow bands in extracts with the intensity of yellow bands obtained with gallic acid standard solutions after spraying with ethanolic DPPH• solution (Table 2). Statistical parameters for calibration using gallic acid are given in Table 4.

Free radical scavenging activities were found to be highly correlated with polyphenolic content (r = 0.86). Some authors claim that there is no correlation between the total phenolic content and the radical scavenging capacity [27], so it was very important to determine if there was a correlation between the total phenolic content and antioxidant capacity in this work. The radical-scavenging capacity of seaweed ethanolic extracts might be mostly related to their phenolic hydroxyl groups. This correlation suggests that although seaweeds may contain other antioxidants such as ascorbic acid and carotenoids, these contribute in a minor way to antioxidant activity. Fatty acids can also attribute to antioxidant activity. For example, palmitic acid was reported to be a more effective free radical scavenger than β-carotene [28]. *n*-Hexadecanoic acid may also contribute to the antioxidant activity [29].We have found that *Phyllospora comosa* (Crayweed), a species of brown algae in the *Seirococcaceae* family, has the highest level of polyphenols and also highest free radical scavenging activity. It also contains small amounts of fucoxanthin. *Phyllospora comosa* contains *n*-hexadecanoic acid and 9,12-octadecadienoic acid as the dominant fatty acids in significantly higher levels than the amount of the other compounds present [30].

Fucoxanthin, the most abundant marine-based carotenoid, has been considered as a potent antioxidant, in terms of its free scavenging activity due to the presence of an unusual allenic double bond (C=C=C) [31] that is believed to be responsible for its higher antioxidant activity [32]. However our study indicates that there are much more potent antioxidants in investigated samples. For example, samples 3 and 11, with higher amounts of fucoxanthin

(Table 5), contain other antioxidants that show higher free radical scavenging activity as measured by intensity of the pale yellow bands after derivatization with DPPH• (Fig. 2b).

Fig. 2.

3.1. Principal component analysis

Principal component analysis (PCA) is an advanced statistical chemometric tool commonly used to observe groupings of objects, outliers, etc. which define the structure of the data set. PCA creates new dimensions of the data and evaluates a reduced number of principal components (independent factors) that describe group of characteristic and partially dependent variables. The aim is to find factors that can explain major variations within the data. In clustering, the objects are grouped based on similarity. Objects in a given cluster should be similar in relation to a number of characteristics that describe the cluster's properties and separate the cluster from the others. The loading plot showed correlation between the original variables and the factor/rotated factor derived from PCA. These numbers represent significant contribution of bioactive compounds, color properties and antioxidant activity to the total variability. Principal component analysis was applied on a data set of 16 algae samples and 675 variables expressed by pixels obtained by HPTLC image analysis. PCA found the similarity and dissimilarity between investigated algae samples. The score plot in Fig. 3 shows the distribution of the samples on the plane described by the first two principal components describe 38.22% and 24.64% of total variability, respectively. First four components describe 82.63% of total variability. There are five clearly separated clusters classified according to chemical composition (Fig. 3a). Algae 2, 3, 11 and 12 formed one separated cluster on lower right hand side of the PC score (cluster 1). These are all brown algae species and they contain significant amounts of fucoxanthin (2.5-11.6 µg/10 µL), together with a wide range of polyphenolic content (10.1-44.1 µg GAE/10 µL) and radical scavenging activity (2.8-13.4 µg GAE/10 µL).

Fig. 3.

Fig. 4.

Generally, brown algae have higher antioxidant potential when compared to red or green algae [33]. Numerous compounds which exhibit antioxidant activity have been isolated from brown algae, most of them being polyphenolic antioxidants [34] .

A group of brown algae samples 1, 13, 15 and 16 form a cluster near the center (right hand side) of the plot (cluster 2). They were found to have low polyphenolic content (0.0-6.9 µg GAE/10 µL), low radical scavenging activities (0.0-1.1 µg GAE/10 µL), and low levels of fucoxanthin (0.8-1.5 µg/10 µL). A small cluster widely separated from the others (on the upper right side of PC score) consists of algae 6 and 14 (cluster 3). These algae have medium levels of polyphenols (18.4-20.4 µg GAE/10 µL), with low free radical scavenging activities (0.4-0.5 µg GAE/10 µL), and fucoxanthin below the level of quantification in both samples. Sample 6 is *Codium fragile* (green algae) and 14 is *Amphibolis antartica* (sea grass). A recent study has found that for seaweed species there is generally no significant difference in their antioxidant activities in winter and summer. However, antioxidant activities of the seagrass species are significantly higher in summer [35]. The cluster (upper left hand side of plot) containing samples 5, 7, 9 and 10 are a mixture of brown and red algae (cluster 4). They all have relatively low polyphenolic content (0.8-10.7 µg GAE/10 µL) and low free radical scavenging activity (0.7-1.1 µg /10 µL). The small cluster (cluster 5) consisting of algae samples 4 and 8 are red algae with relatively low polyphenolic content (4.6-15.6 µg GAE/10 µL) and low free radical scavenging activity (0-3.1 µg/10 µL). Algae 8 has a small amount of fucoxanthin, very close to the level of quantification (0.8 µg/10 µL) while algae 4 has no observable fucoxanthin.

The loading plots (Figs. 3b and c) enable identification of the most important phenolic compounds for discriminating between the algae clusters. PC1 was highly contributed by compounds with R_F values at 0.31, 0.50, 0.57, 0.65, and 0.86, while PC2 was positively contributed by R_F values at 0.19, 0.56 and 0.65. The compound with an R_F value of 0.19 was recognized as fucoxanthin. In particular, the compound with an R_F value of 0.65 together with fucoxanthin could be used as potential markers for distinguishing between algae 1, 10, 13, 15 and 16 and other algae samples.

Conclusion

HPTLC combined with post-derivatization DPPH• assay and ferric chloride assay, were used to successfully screen marine algae samples for antioxidant activity and polyphenolic content. These assays are based on their ability: (a) to scavenge non biological stable free radical

(DPPH•); or (b) to chelate Fe^{3+} ions. The investigated brown algae samples were found to be rich in non-polar and moderately polar compounds and contain phenolic compounds with significant free radical scavenging activity. Strong, positive, and significant correlations between total phenolic content and DPPH• radical scavenging activity, showed that phenolic compounds, including flavonoids, are the main contributors of antioxidant activity in these algae species. Fucoxanthin, the most abundant marine-based carotenoid, has been considered as a potent antioxidant, in terms of its free scavenging activity due to the presence of an unusual allenic double bond (C=C=C) [31] that is believed to be responsible for its higher antioxidant activity [32]. However our study indicates that there are much more potent antioxidants in investigated samples. For example, samples 3 and 11, with higher amounts of fucoxanthin (Table 5), contain other antioxidants that show higher free radical scavenging activity as measured by intensity of the pale yellow bands after derivatization with DPPH•. Brown algae samples *Phyllospora comosa* and *Zonaria angustata* were found to have significantly higher antioxidant activity than the other algae samples screened. This make them ideal candidates for further investigation in medical, dietary supplement, and/or cosmetic formulation applications.

The method developed in this work provide an edge over existing methods used to screen algae for antioxidant activity, as it is possible to quantify antioxidant activity for individual compounds in the extract mixtures. This work also demonstrates the flexibility and versatility of a standard HPTLC system as a useful tool in the drug discovery process. The method developed in this work can also be used for the bioassay-guided isolation of unknown natural antioxidants in extract mixtures and their subsequent identification of components by utilizing post-chromatographic mass spectroscopy analysis techniques.

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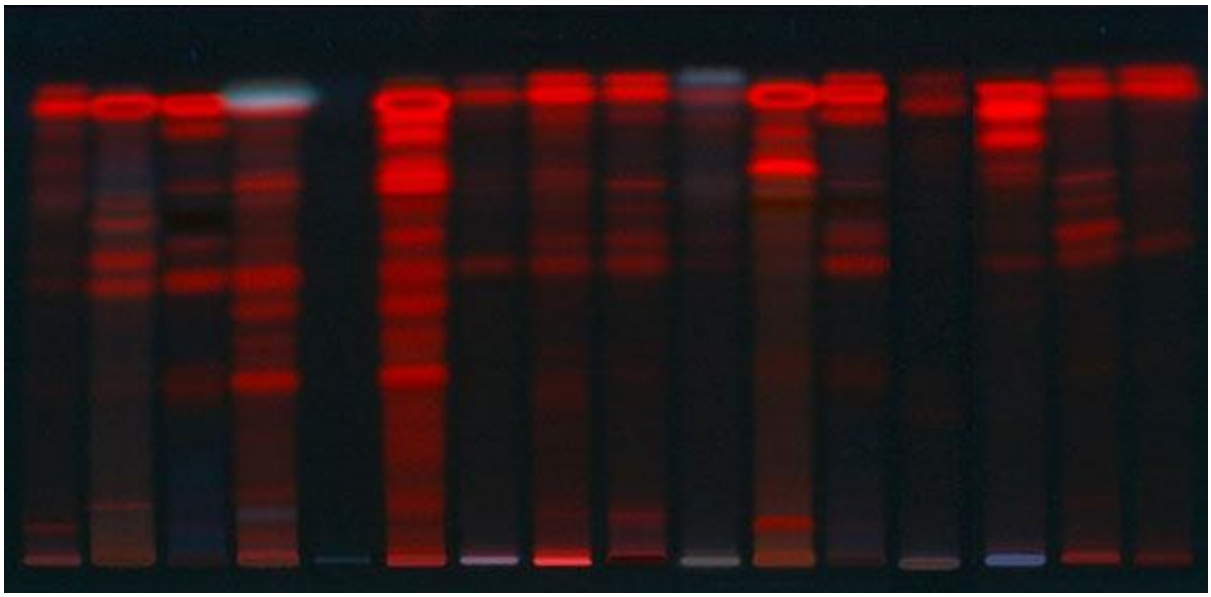
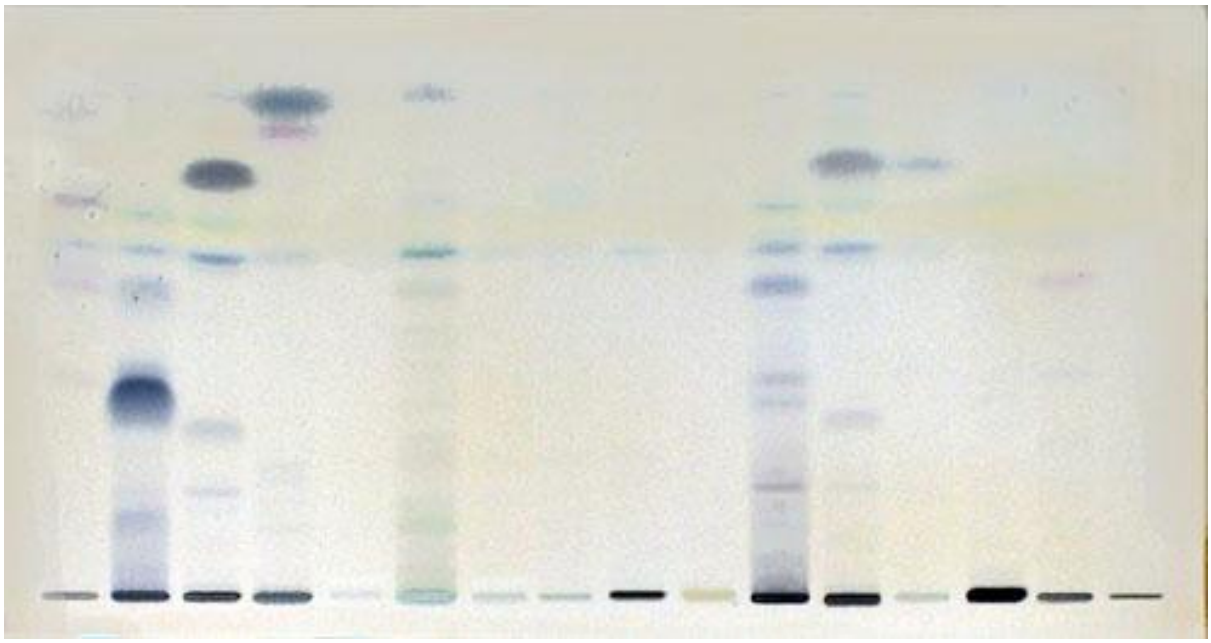
Figure captions





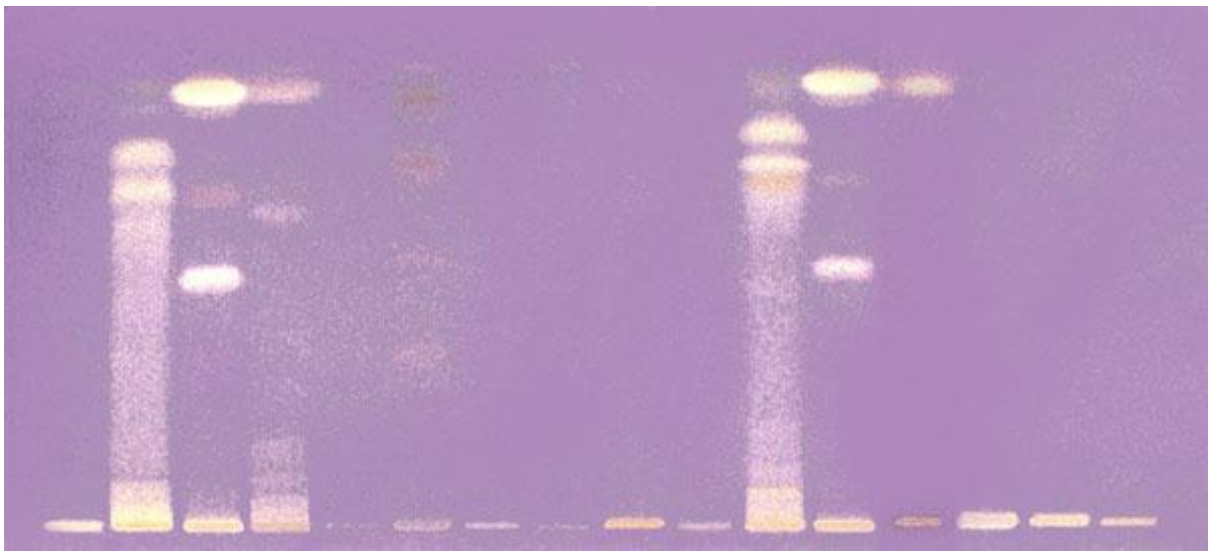


Fig. 1. HPTLC fingerprints of algal extracts (samples 1-16 from left to right) on normal phase HPTLC plates before derivatization and under 366 nm (a) and after post-chromatographic derivatization with (b) $FeCl_3$ and (c) DPPH•. Mobile phase, hexane: ethyl acetate: acetic acid (20:10:1). Fig. 1(a) photos taken under 366 nm, and Figs. 1(b) and (c) with white light above.

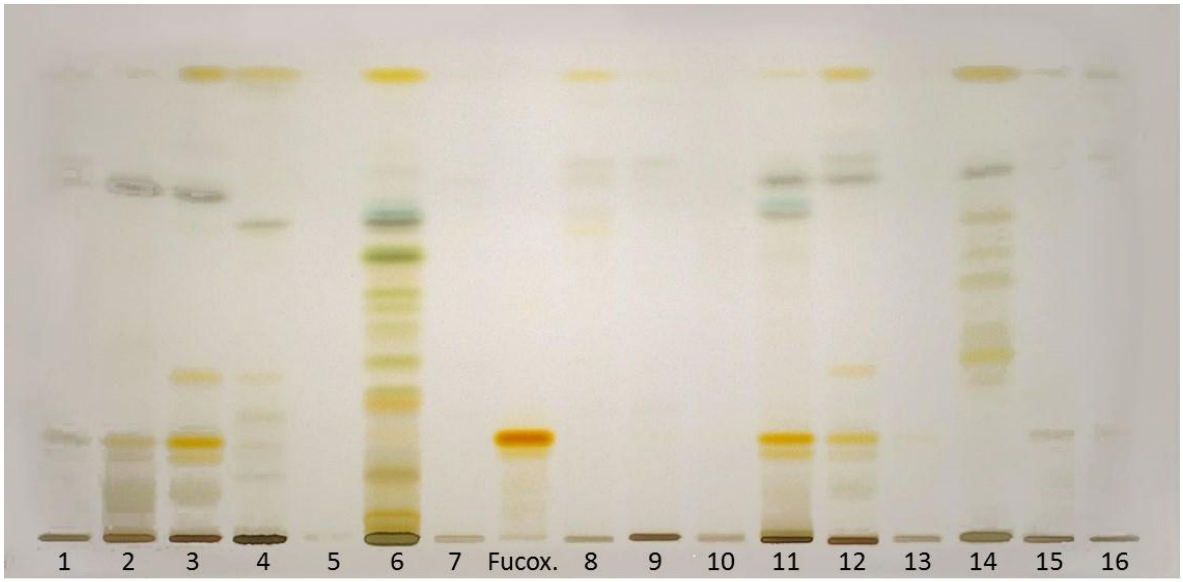
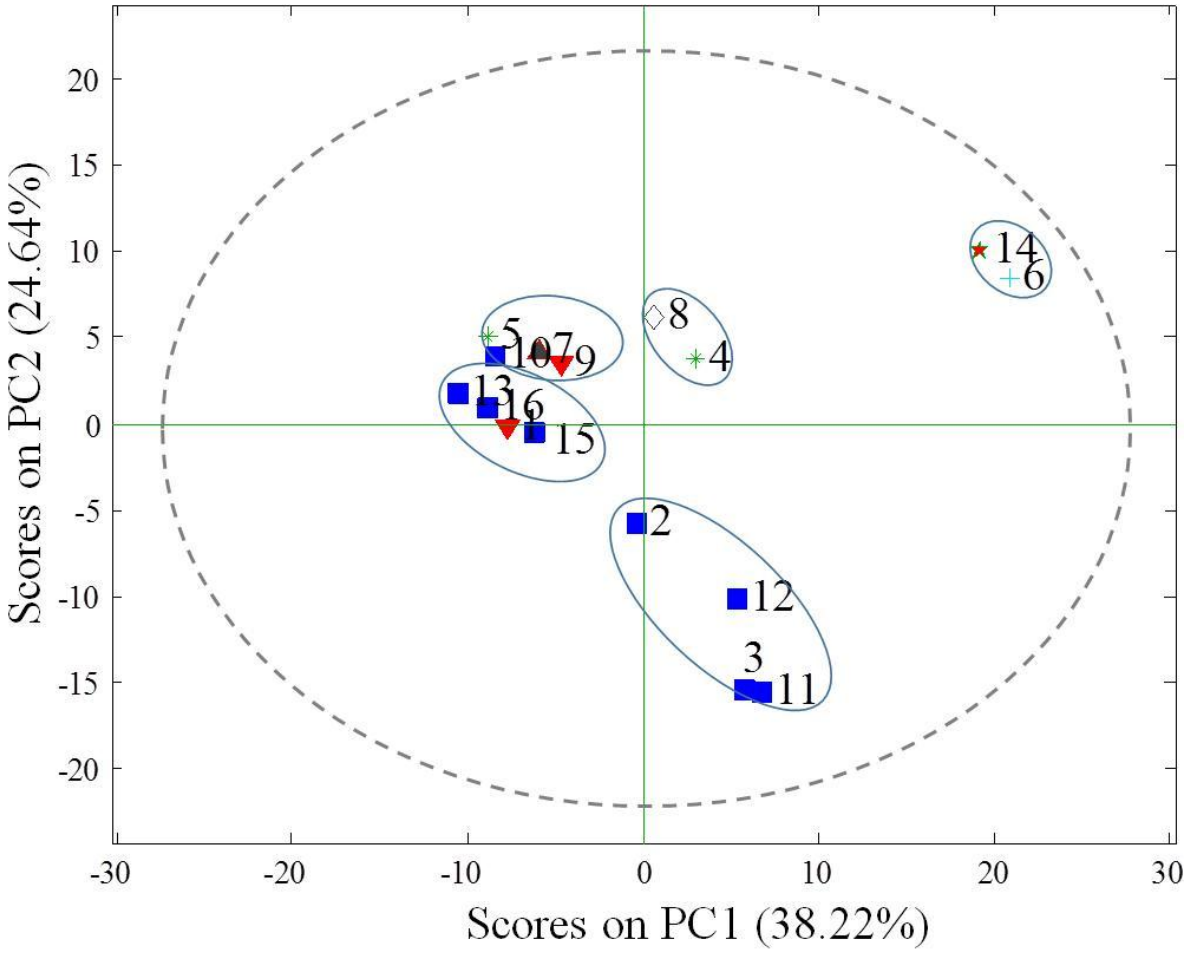
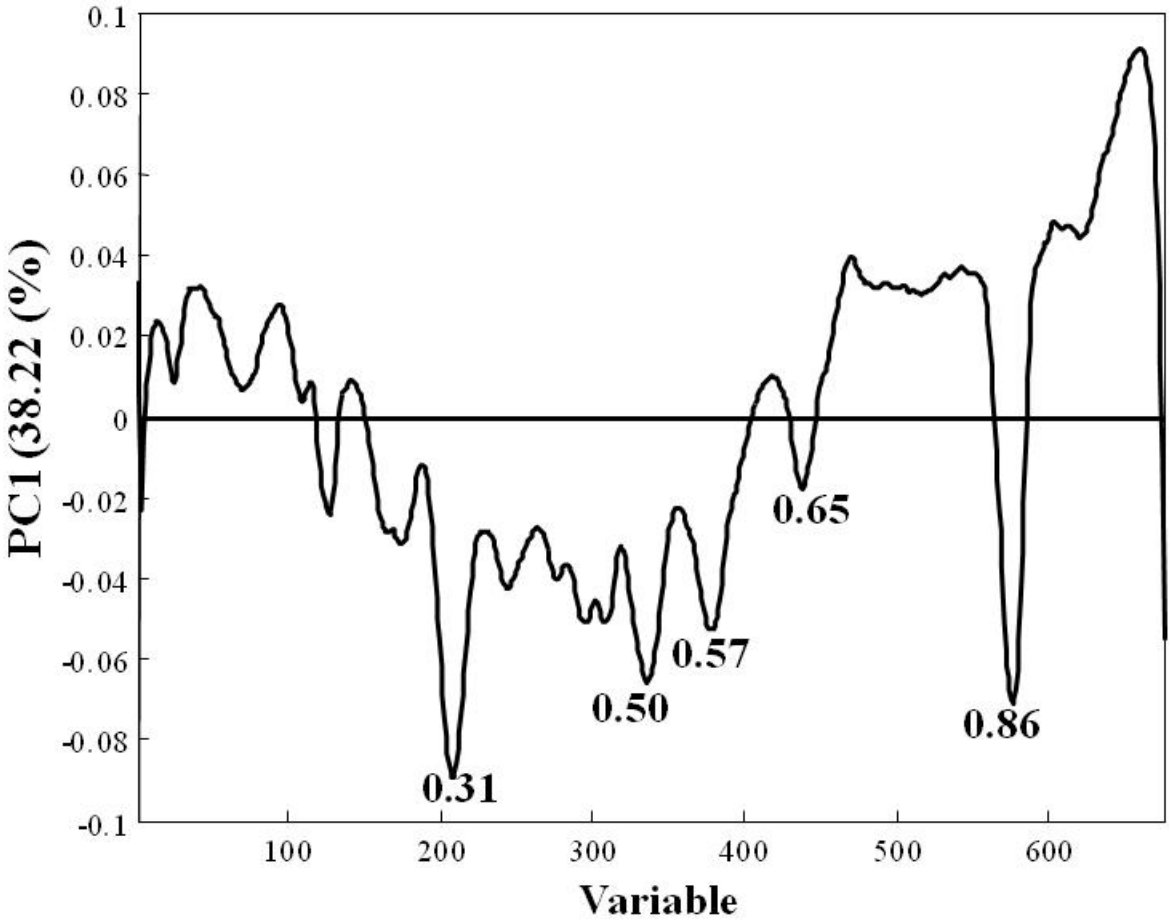


Fig. 2. HPTLC fingerprints of algal extracts (samples 1-16 from left to right) and fucoxanthin standard on normal phase HPTLC plates before derivatization and under white light. Mobile phase, hexane: ethyl acetate: acetic acid (20:10:1).





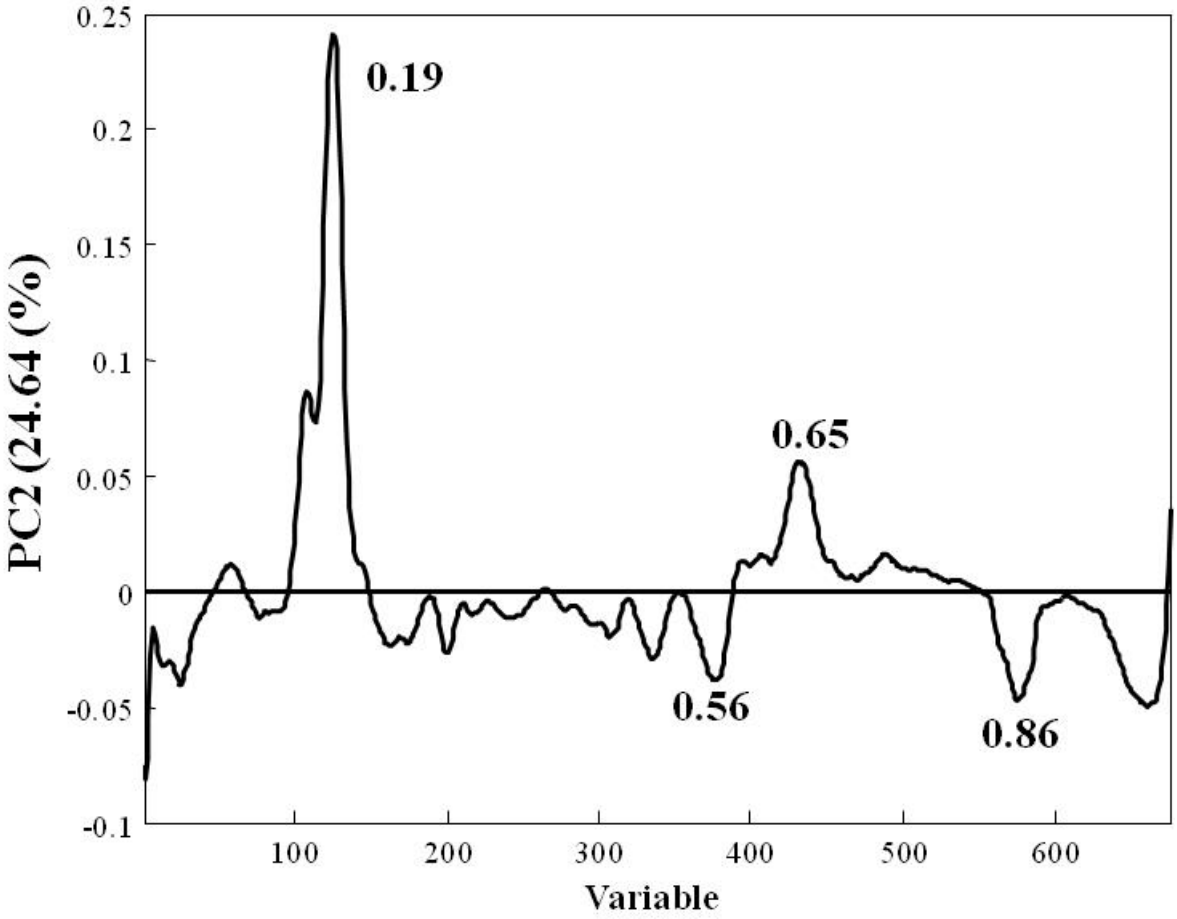
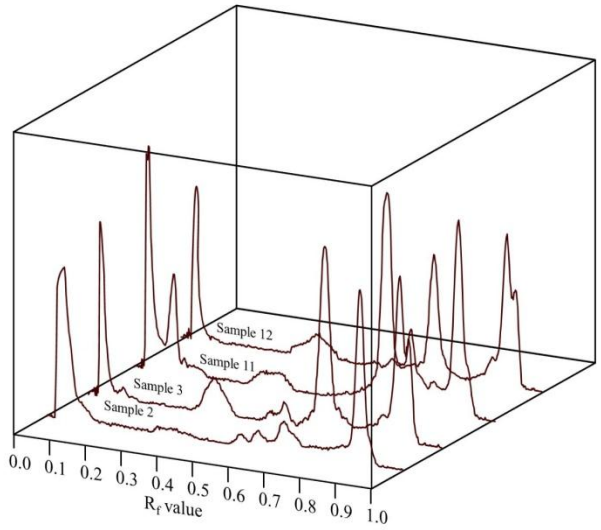
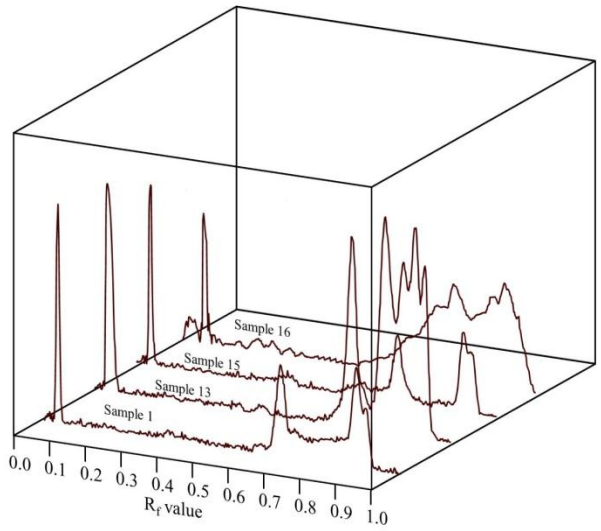
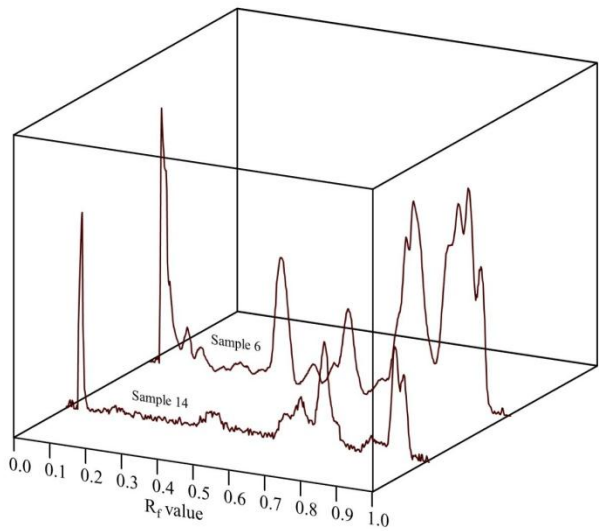


Fig. 3. Principal component analysis: (a) bi-plot for object scores of the first two principal vectors of 16 algae samples (score plot PC1 versus PC2) with groupings are indicated by circles; (b) loading plot PC1; (c) loading plot PC2.







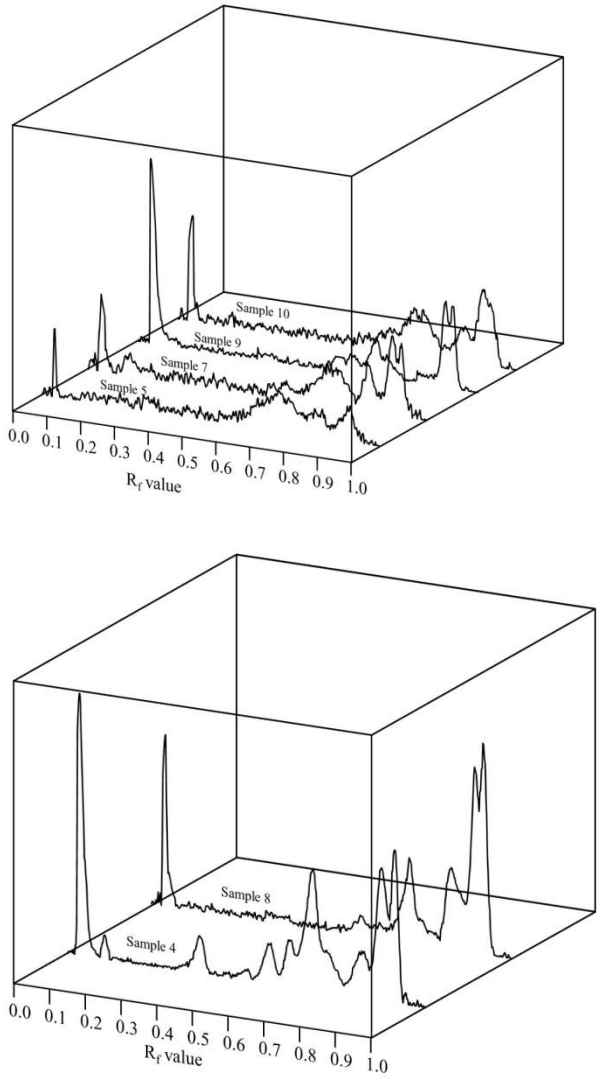


Fig. 4. Superimposed chromatograms of algae samples that are in the same cluster. (a) cluster 1 (samples 2, 3, 11 and 12); (b) cluster 2 (samples 1, 13, 15 and 16); (c) cluster 3 (samples 6 and 14); (d) cluster 4 (samples 5, 7, 9 and 10); (e) cluster 5 (samples 4 and 8).